

AKH 34

CLAIMS

- 5 1. Deacetoxycephalosporin C synthase (DAOCS) having a structure designated by the X-ray co-ordinates of structure A or structure B herein.
2. DAOCS in the form of a complex with a metal, e.g. iron or
10 lead, and optionally in the presence of a substrate and/or a substrate analogue or inhibitor, having a structure designated by the X-ray co-ordinates herein.
3. DAOCS as claimed in claim 2, wherein the substrate is
15 penicillin N, penicillin G, 2-oxoglutarate or dioxygen, and the inhibitor is selected from N-oxalylamino acids, pyridine-carboxylates and nitrous oxide.
4. Use of the three-dimensional structure of DAOCS for the
20 modification of DAOCS or other related 2-oxoglutarate dependent enzyme.
5. Use as claimed in claim 4, wherein the related 2-oxoglutarate dependent enzyme is DACS, DAOC/DACS or the oxygenase enzyme involved in the introduction of the 7 α -methoxy group into cephamycin C.
- 25 6. Use as claimed in claim 5 for the modification of DAOCS, DACS or DAOC/DACS such that they accept unnatural substrates more efficiently than the wild type enzymes.

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7. Use as claimed in claim 5 for the modification of DAOCS, DACS, DAOC/DACS such that they convert natural substrates to pharmaceuticals or useful intermediates in the preparation of pharmaceuticals.

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8. Use as claimed in claim 6 wherein the unnatural substrates are penicillins including penicillin G, penicillin V, 6-aminopenicillanic acid, amoxycillin, or penicillins with a phenyl glycine or p-hydroxyphenyl glycine side chain.

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9. Use as claimed in claim 6 wherein the unnatural substrate is a cephalosporin.

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10. Use as claimed in claim 6 wherein the unnatural substrate is an amino acid, including the proteinogenic amino acids, or a peptide.

11. Use as claimed in any one of claims 6-8, wherein penicillin G, penicillin V, another unnatural substrate or penicillin N is converted to a cephalosporin or exomethylene cephalosporin.

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12. An enzyme having significant (as herein defined) sequence similarity to DAOCS wherein the side chain binding site of penicillin N or DAOC is modified and at at least one of the following sites at least one amino acid residue is changed to another amino acid residue or is deleted:

25 Thr72, Arg74, Arg75, Glu156, Leu158, Arg160, Arg162, Leu186, Ser187, Phe225, Phe264, Arg266, Asp301, Tyr302, Val303, Asn304; and/or at least one additional amino acid residue is inserted within the region 300-311; provided that other residues interacting with the above may be changed in order to accommodate the change in one of the above;

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wherein the modifications:

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- permit the enzyme to accept unnatural substrates; and/or
- enable the enzyme to produce unnatural products; and/or
- enhance the ability of the enzyme to produce useful products.

5 13. An enzyme having significant (as herein defined) sequence similarity to DAOCS wherein the penicillin/cephalosporin binding site of penicillin N or DAOC is modified and at least one of the following amino acid residues is changed or deleted: Ile88, Arg160, Arg162, Phe164, Met180, Thr190, Ile192, Phe225, Pro241, Val245, Val262, Phe264, Asn 304, Ile305, Arg306, Arg307; and/or at least one additional amino acid residue is inserted within the region 300-311; provided that other residues interacting with the above may be changed in order to accommodate the change in one of the above;

wherein the modifications:

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- permit the enzyme to accept unnatural substrates; and/or
 - enable the enzyme to produce unnatural products; and/or
- enhance the ability of the enzyme to produce useful products.

14. An enzyme according to claim 12 or claim 13 which is a
20 mutant of DAOCS or DACS or DAOC/DACS.

15. An enzyme as claimed in any one of claims 12-14, wherein both the side chain and the penicillin/cephalosporin binding sites of penicillin N or DAOC are modified and at least one of the residues
25 specified in claims 12 and 13 is changed or deleted.

16. An enzyme as claimed in any one of claims 12-15, wherein two or more complementary mutations are introduced to create or delete a binding interaction, including H-bonds, electrostatic, or hydrophobic
30 interactions.

17. A gene encoding for the enzyme of any one of claims 12-16.

18. A micro-organism capable of expressing the gene of claim 17
5 under fermentation conditions.

19. Use of micro-organisms of claim 18 for the production of
beta-lactams of the penicillin or cephalosporin (including cepham) families.

20. Use as claimed in claim 19 wherein the micro-organism
10 contains another modified enzyme of the penicillin and cephalosporin
biosynthesis pathway including isopenicillin N synthase,
amidohydrolase/acetyltransferase, or L-delta-(aminoadipoyl)-L-cysteine-D-
valine (ACV) synthetase.

21. A method which comprises using the three-dimensional
15 structure of DAOCS for determining or predicting the structure of another
related 2-oxoglutarate dependent enzyme or related enzyme not from the
penicillin and cephalosporin biosynthesis pathway, and using the structural
information so obtained for modifying the other enzyme or for designing an
20 inhibitor for the other enzyme; wherein the said other related enzyme is
modified, by deletion or addition or alteration; at one or more of the sites
defined in claim 12 or claim 13; or using the following information for the
design of an inhibitor: Asp185, His183 and His243 act as ligands to the
iron; Arg258 and Ser260 and the Fe bind the 2-oxoglutarate; Met180,
25 Phe225, Leu31 and Val245 are close to the iron binding site; Tyr33,
Arg160, Arg162, Phe164, Ile192, Gln194, Leu204, Leu223, Leu215 are
important for the construction of the part of the active site binding 2-
oxoglutarate; and Arg160 and Arg162 are important for binding an amino
acid or peptide derived substrate.

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22. A method as claimed in claim 21 wherein the said other related 2-oxoglutarate dependent enzyme or related enzyme is 1-aminocyclopropane-1-carboxylate oxidase, gibberellin C-20 oxidase, flavone synthase, flavanone 3 β -hydroxylase, hyoscyamine 6 β -hydroxylase, 5 prolyl 4-hydroxylase, prolyl 3-hydroxylase, aspartyl hydroxylase, lysyl hydroxylase, proline hydroxylases, γ -butyrobetaine hydroxylase, enzymes in herbicide resistance mechanisms, clavamate synthase, an oxygenase enzyme involved in the biosynthesis of carbapenems, the so called 10 ethylene forming enzyme from *Pseudomonas syringe*, p-hydroxyphenylpyruvate dioxygenase, and an oxygenase enzyme involved in the oxidation of phytol in human liver peroxisomes.

23. A method as claimed in claim 21 or 22, wherein the said 15 other related enzyme is prolyl 4-hydroxylase, prolyl 3-hydroxylase, aspartyl hydroxylase, or lysyl hydroxylase and the inhibitor is to be used for the treatment of human diseases including fibrotic diseases including liver cirrhosis and arthritis.

24. A method as claimed in claim 21 or 22, wherein the said 20 other related enzyme is p-hydroxyphenylpyruvate dioxygenase and the inhibitor is to be used in the treatment of certain genetic disorders.

25. A method as claimed in claim 21 or 22, wherein the said 25 other related enzyme is involved in herbicide resistance and the information is to be used to design new herbicides to overcome the problem of resistance.

26. An enzyme as claimed in any one of claims 12 to 16, which 30 has modifications at at least two of the said amino acid residues.

add a1)

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